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PARASITOLOGY

Evaluation of a PCR Method for Detection of *Entamoeba polecki*, with an Overview of Its Molecular Epidemiology

Christen Rune Stensvold,[a] Jadwiga Winiecka-Krusnell,[b] Tore Lier,[b] Marianne Lebbad[b]

[a]Statens Serum Institut, Copenhagen, Denmark
[b]Public Health Agency of Sweden, Solna, Sweden

ABSTRACT *Entamoeba polecki* is a parasite of human and nonhuman primates, other mammals, and birds. Due to overlapping morphological features, cysts of *E. polecki* may be confused with those of other *Entamoeba* species commonly found in human fecal samples, including immature cysts of *Entamoeba histolytica*. Although the presence of *E. polecki* in human *Entamoeba*-positive stool samples may be rare, its prevalence is likely underestimated due to such confusion. Here, we give examples of diagnostic approaches applied so far and summarize data on the molecular epidemiology of *E. polecki*, including host specificity and phylogeography. Moreover, we evaluate a novel diagnostic conventional PCR developed for the screening of fecal samples for *E. polecki*. The assay was highly sensitive and specific when used on genomic DNA extracted directly from stool and Swedish wastewater samples. The PCR enabled the identification of all four subtypes (ST1 to ST4) of *E. polecki* by PCR product sequencing. Most (23/28) subtyped *E. polecki*-positive samples detected in patients in Sweden between 2002 and 2015 reflected colonization by ST4 and were seen in travelers/foreigners. Two and three human cases of ST2 and ST3, respectively, were also detected. Subtypes 1, 2, and 3 were detected in 3/21 wastewater samples, suggesting local endemicity of these *E. polecki* subtypes; interestingly, ST4 was not detected in wastewater. In conclusion, the current PCR assay enables simple and cost-effective screening of fecal and wastewater samples for *E. polecki*. Human cases of *E. polecki* appear to involve primarily ST4, while *E. polecki* detected in wastewater may be primarily of animal origin.

KEYWORDS diagnosis, gut, parasite, public health, protozoa

Several species of *Entamoeba* are capable of infecting or colonizing the human gastrointestinal tract from the cavum oris to the rectum. *Entamoeba histolytica* is known for its invasive properties and should be distinguished from other entamoebas for treatment purposes, with most other entamoebas found in human stool samples considered nonpathogenic.

The morphological diagnosis of *E. histolytica* infection is a renowned challenge in the clinical microbiology laboratory. Several species of *Entamoeba* share morphological features that appear similar, and so it is not possible to tell apart cysts of *Entamoeba dispar* from those of *E. histolytica* by simple light microscopy of fecal concentrates. The cardinal diagnostic hallmarks for the differentiation of entamoebas include cyst size, the number of nuclei, the appearance of the peripheral chromatin, the presence/absence and appearance of chromatoid bars, the size of the karyosome, the presence/absence of inclusion bodies, etc.; however, some of these hallmarks may not always be discernible by light microscopy of fecal concentrates, and the existence of mature as well as immature cysts allows for a continuum of numbers of nuclei, which blurs the diagnostic criteria. Immature cysts of *E. histolytica* and *E. dispar* may have only one nucleus, and even in more mature cysts, only one nucleus may be discernible by

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Address correspondence to Christen Rune Stensvold, run@ssi.dk.

microscopy. Hence, it may be almost impossible to differentiate such cysts from those of *Entamoeba polecki*, a uninucleated species of *Entamoeba*, the cysts of which are similar in size to those of *E. histolytica* and *E. dispar*. Compared with *E. histolytica*/*E. dispar*, *E. polecki* has been much more rarely reported in surveys of intestinal parasites; however, the lack of data on this species may indeed be related to the possibility that it is confused with other entamoebas. Furthermore, in many laboratories, PCR-based diagnostics for *Entamoeba* only include the detection of *E. histolytica* and, sometimes, *E. dispar*, and so *E. polecki* would obviously be missed.

Parallel to the situation for *Blastocystis* back in 2007 (1), a novel terminology for *E. polecki* was suggested in 2012 (2). According to this terminology, *E. polecki* comprises four subtypes (ST1 to ST4), all of which have been found in humans (2). *E. polecki* ST2 was previously known as *E. chattoni*, whereas *E. polecki* ST3 has occasionally been referred to as *E. struthionis*. Along with ST2, ST4 has been found exclusively in primates, and ST4 appears to be the most common subtype in humans and with the widest geographical range (2). Therefore, there might be a potential for transmission between animal and human hosts for most of the subtypes depending on the level of cryptic host specificity, which remains largely unexplored for this species, although preliminary data suggest high intrasubtype genetic similarity (2).

Even though some early reports suggested symptoms related to *E. polecki* (3, 4), it is our impression that there is broad consensus that *E. polecki* is nonpathogenic. Meanwhile, investigators recently reported a link between *E. polecki* ST3 and invasiveness in postmortem studies of pigs (5), a finding which remains to be further clarified and confirmed. Nevertheless, it remains clear that well-designed diagnostic tools are critical to expanding our knowledge on the epidemiology and clinical and public health significance of *E. polecki*.

Here, we present data from an evaluation of a conventional PCR for the specific and sensitive detection and molecular characterization of *E. polecki*. Moreover, we review data available in GenBank and in the scientific literature to provide an overview of what is known with regard to the molecular epidemiology of *E. polecki* infections.

MATERIALS AND METHODS

Data collection. DNA sequences representing *E. polecki* nuclear small subunit (SSU) ribosomal genes along with metadata were retrieved from GenBank, regardless of sequence length and sequence region. Moreover, a search on PubMed was carried out to identify studies with information on *E. polecki*; the names *Entamoeba polecki*, *Entamoeba chattoni*, and *Entamoeba struthionis* were entered in the search to ensure an exhaustive literature search.

Samples. For several years, human stool samples suspected positive for *E. histolytica/E. dispar* have been routinely referred from different local Swedish laboratories to the Public Health Agency of Sweden (http://www.folkhalsomyndigheten.se/about-folkhalsomyndigheten-the-public-health-agency-of-sweden/) for PCR-based species-specific diagnosis of *E. histolytica* and *E. dispar* infections. All samples received for PCR analysis are also investigated by microscopy on a routine basis. In the parasitology laboratory at the Public Health Agency of Sweden, uninucleated cysts of *Entamoeba* (UNE) in which the appearance of the nucleus, inclusions, and chromatoidal bodies are suggestive of *E. polecki* are defined as potential *E. polecki* cysts. In the present study, 18 samples from 18 patients that were microscopy-positive for UNE were included; these samples were analyzed between 2010 and 2015, and no amplification product was obtained by routine *E. histolytica*/*E. dispar* PCR (6) for any of the samples (Table 1).

In addition to the 18 DNA samples from patients suspected of carrying *E. polecki*, 6 DNA samples from pig stools were included, 2 with PCR-confirmed *E. polecki* ST1 and ST3 (7) and 4 with unknown *E. polecki* status (Table 1). To evaluate the specificity of the primers, DNAs from seven other *Entamoeba* species were tested. Three DNAs were from cultures (*E. histolytica*, *Entamoeba gingivalis*, and *Entamoeba moshkovskii*), two from cyst gradients (8) (*E. dispar* and *Entamoeba* sp. strain RL1 [2, 7, 9] from a ruminant), and the remaining from *Entamoeba*-positive stool samples (*Entamoeba coli*, *E. histolytica/E. dispar*, and *Entamoeba hartmanni*). Three other stool parasites were also included in the evaluation, *Blastocystis* sp., *Endolimax nana*, and *Iodamoeba bütschlii*. For further testing of primer specificity, DNA from 21 influent wastewater samples, collected from eight different Swedish treatment plants, was included.

DNA extraction. DNA was extracted directly from human stool specimens using a QIAamp DNA minikit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. Prior to extraction, the cysts were disrupted in lysis buffer (8) using a Mini-beadbeater (Biospec Products Inc., Bartlesville, OK, USA). For the wastewater specimens included for primer specificity testing, the cysts were isolated using a sucrose gradient prior to DNA extraction. Briefly, each wastewater sample was shaken to ensure a homogenous sample, and 200 ml was filtered into a 200-ml Falcon bottle through four layers of gauze. The samples were centrifuged at 1,700 × *g* (3,000 rpm) for 10 min. The supernatants were decanted, leaving approximately 10 ml of each sample. The material was transferred to a 10-ml centrifuge tube

TABLE 1 Overview of patient, pig, and wastewater samples included in the present study[a]

Sample ID[b]	Host/source	Year of diagnosis	Travel history	Microscopy result	PCR for *E. histolytica*/*E. dispar*	*E. polecki* subtype
UNE15	Human	2010	Somalia/Ethiopia	UNE[c]	Neg	ST4
UNE16	Human	2011	NA[d]	UNE	Neg	ST4
UNE17	Human	2011	Afghanistan	UNE	Neg	ST4
UNE18	Human	2012	Kenya (2010)	UNE	Neg	ST4
UNE19	Human	2012	Spain	UNE	Neg	ST3
UNE20	Human	2012	NA	UNE, *Endolimax nana*	Neg	ST4
UNE21	Human	2012	NA	UNE	Neg	NP[e]
UNE22	Human	2013	NA	UNE	Neg	ST4
UNE23	Human	2014	NA	UNE, *Chilomastix mesnili*	Neg	ST4
UNE24	Human	2014	NA	UNE, *Blastocystis* sp.	Neg	ST2
UNE25	Human	2014	NA	UNE, *E. nana*, *Blastocystis* sp.	Neg	ST4
UNE26	Human	2014	Italy/Somalia	UNE, *E. nana*	Neg	ST4
UNE27	Human	2015	Eritrea/Somalia	UNE, *E. nana*	Neg	ST4
UNE28	Human	2015	Eritrea/Somalia	UNE, *E. nana*	Neg	ST4
UNE29	Human	2015	Eritrea/Somalia	UNE, *E. nana*, *Blastocystis* sp., *Trichuris trichiura*, *Hymenolepis nana*	Neg	ST4
UNE30	Human	2015	Eritrea/Somalia	UNE, *E. nana*, *C. mesnili*	Neg	ST4
UNE31	Human	2015	Eritrea/Somalia	UNE, *E. nana*, *Giardia intestinalis*, *H. nana*	Neg	ST4
UNE32	Human	2015	Kenya?	UNE, *Blastocystis* sp.	Neg	ST2
A83	Wastewater, Sweden	NA	NA	NP	NP	ST3
A88	Wastewater, Sweden	NA	NA	NP	NP	ST2
A95	Wastewater, Sweden	NA	NA	NP	NP	ST1, ST3
D209	Pig, Sweden	NA	NA	NP	NP	ST1, ST3
D210	Pig, Sweden	NA	NA	NP	NP	ST1, ST3
D211	Pig, Sweden	NA	NA	NP	NP	ST3
G17	Pig, Sweden	NA	NA	NP	NP	ST1, ST3
ST1	Pig, UK	NA	NA	NP	NP	ST1, ST3
ST3	Pig, UK	NA	NA	NP	NP	ST1, ST3

[a]All samples were positive for *E. polecki* by specific PCR.
[b]ID, identifier.
[c]UNE, uninucleated cysts of *Entamoeba*.
[d]NA, information not available/applicable.
[e]NP, not performed.

and centrifuged at 1,700 × *g* for 10 min, and the supernatants were discarded. The sediments were washed again with Milli-Q water for 10 min at 1,700 × *g*. The supernatants were discarded. The sediments were processed by a sucrose gradient, and DNA was extracted from separated cysts as previously described (8).

PCR and sequencing. PCR amplification was performed using the primers UNINUC_400F (5′-AGG TAG TGA CGA TAA TTA ATA G-3′) and UNINUC_1050R (5′-TCC TGA ATA AGA GAG TAA CAA T-3′) targeting the nuclear SSU rRNA gene, with expected PCR products comprising ≈600 bp. Our intention was to design diagnostic primers, and oligonucleotides potentially meeting this criterion were identified based on *in silico* analysis of an alignment of relevant *Entamoeba* sequences available in GenBank, including complete nuclear SSU ribosomal DNA sequences representing the four subtypes (AF149913 [ST1], AF149912 [ST2], AJ566411 [ST3], and FR686357 [ST4]), and PrimerBlast (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). PCRs were carried out in total volumes of 20 μl, using Maxima Hot Start PCR master mix (Thermo Scientific), 0.5 μM each primer, and 1 to 2 μl of extracted DNA. Reaction conditions included an initial denaturation at 95°C for 4 min, followed by 35 to 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, followed by a final extension step at 72°C for 7 min.

Sequencing and phylogenetic analysis. The amplified DNAs were purified using ExoSAP-IT (Thermo Fisher) and subjected to sequencing (BigDye Chemistry; Applied Biosystems) using the primers employed in the PCR. The DNA sequences were aligned, edited, and compared with sequences in GenBank using the Basic Local Alignment Search Tool (BLAST; NCBI) (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The phylogenetic analysis was performed using MEGA7 (10).

Accession number(s). The sequences were deposited in the NCBI database (accession no. MG747649 to MG747668).

RESULTS

All 18 study samples that had tested positive by microscopy for potential *E. polecki* cysts and negative for *E. histolytica* and *E. dispar* by PCR were positive by the *E. polecki* PCR (Table 1) as well as the six samples from pigs. Three of the 21 screened wastewater samples produced bands of the appropriate size, while all other wastewater samples produced no bands; hence, no unspecific amplification was seen (data not shown). No

PCR products were observed for any of the DNAs representing other *Entamoeba* species/stool parasites (data not shown).

Sequencing confirmed the diagnosis of *E. polecki* and also enabled the identification of *E. polecki* subtypes. Most of the patients had ST4 ($n = 14$), while ST2 was seen in two cases and ST3 in one case (Table 1). None of the patient samples tested positive for ST1. One PCR-positive patient sample (UNE21) was not subjected to sequencing, as the material had gone missing.

Sequencing of the amplicons from the pig samples confirmed mixed infections with ST1 and ST3 in the samples with known STs, while one of the previously untested pig samples gave a clear ST3 sequence, suggesting single-ST colonization; the remaining three samples contained mixtures of ST1 and ST3 (Table 1). The wastewater sample from a major urban area was positive for ST2; the two samples from a wastewater treatment plant in the countryside contained ST3 and a mixture of ST1 and ST3 (Table 1).

In our PubMed search, we were able to identify 72 articles that included one or more of the names *Entamoeba polecki*, *Entamoeba chattoni*, and *Entamoeba struthionis*; only 30 articles had included one or more of these names in the title of the article. A total of 21 studies had used DNA-based methods for the detection of *E. polecki*, and of these, 10 were linked to *E. polecki* sequences deposited in GenBank (see Table S1 in the supplemental material). Six GenBank sequences were not linked to publications, five of which were from a study in Cameroon. We were able to identify 69 verified partial or complete *E. polecki* nuclear ribosomal DNA (rDNA) sequences in GenBank, including the 20 sequences generated in the present study (Table 1 and Table S1).

DISCUSSION

Entamoeba polecki was described in pigs and humans for the first time by Prowazek in 1912, and 2 years later, Swellengrebel reported on *E. chattoni* in nonhuman primates (11). Over the next many decades, microscopy was the mainstay of detecting and identifying uninucleated cysts of *Entamoeba* in samples of human and nonhuman origin.

Using PCR and sequencing, Verweij et al. (12) demonstrated the existence of four closely related genetic types of uninucleated amoebas with the potential to infect humans, only two of which had been known previously as *E. polecki* (now ST1) and *E. chattoni* (now ST2). One of the two newer lineages, which is now referred to as ST3, was later referred to as *E. struthionis* by Ponce Gordo et al. (13). Owing to the absence of consistent morphological differences among the four lineages and limited host specificity, it was later suggested to refer to these four lineages as *E. polecki*, subtypes 1 to 4 (2).

On the basis of the experiments and observations in the present study, the *E. polecki* PCR introduced here might be applicable as an *E. polecki* diagnostic PCR, with the option of sequencing the amplicons for information on subtype. The fact that only specific bands were produced when applied to DNA from wastewater samples indicates that the primers are highly specific and that the assay in general is robust and reliable as a diagnostic PCR.

Our main concern is the fact that no DNA from *Entamoeba suis* was available for testing. Like *E. polecki*, *E. suis* also produces uninucleated cysts, and DNA-based methods may be critical for differentiating the two in fecal samples from pigs. It therefore remains unknown whether the PCR remains specific in the presence of *E. suis* DNA. However, *in silico* analysis suggests that *E. suis* should not be amplified by the primer set. Nevertheless, until an evaluation of the primers against *E. suis* has been performed, we recommend sequencing PCR products obtained from fecal DNA from pigs for confirmation.

The sequencing of the PCR products was usually straightforward, and both primers worked well as sequencing primers. Unilateral sequencing was usually sufficient in single-subtype infections; for deciphering the subtypes involved in mixed subtype infections, bilateral sequencing may be required, with examining the DNA sequence for

the presence of double peaks (confirmed on both strands) or other features that indicate the presence of more than one subtype.

In 2003, a reverse line blot hybridization assay was introduced, enabling the detection and differentiation of *Entamoeba* species even in cases of mixed subtypes (14). The assay relied on the hybridization of PCR products generated using genus-specific primers and species-specific probes and was able to detect and identify all four ribosomal lineages of *E. polecki*. This assay was later used for screening purposes in studies of humans and nonhuman primates by Levecke et al. (15, 16), who confirmed the applicability of the assay in terms of detecting and differentiating mixed-subtype colonizations.

There are five published studies with data pertaining specifically to *E. polecki* ST1 sequences submitted to GenBank, comprising a total of eight sequences. An ST1 sequence was recently submitted to GenBank as part of a hitherto unpublished study on metagenomic analysis of material from what appeared to be an anaerobic reactor with farm slurry (see Table S1 in the supplemental material). Despite the limited number of observations, no less than three host species have been identified so far for ST1, namely, *Sus scrofa*, *Homo sapiens*, and *Rhea americana*, indicating a limited host specificity. The lack of host specificity could suggest that ST1 might be more common than anticipated on the basis of the available scientific literature. It should be noted that only two cases of human ST1 carriage have been documented so far (2, 12). Meanwhile, ST1 appears to have a geographically wide distribution, with examples of identification in Asia, North America, and Europe (Table S1).

Six studies (the present study included) involving 10 ST2 sequences are available (Table S1). The two sequences, KJ149294 and KJ149295, were originally interpreted as ST4 by Regan et al. (17); however, BLAST and phylogenetic analyses show that these sequences represent ST2, the species formerly known as *E. chattoni* (indeed, the sequences were submitted to GenBank under the name *Entamoeba chattoni*). The misinterpretation might stem from the lack of resolution and representative sequences in the phylogenetic analysis included in the article. Infections by ST2 appear to be limited to primates, ranging in the host spectrum from monkeys (17, 18) and chimpanzees (19) to humans (reference 12 and the present study), and ST2-positive hosts have been found mainly in Asia, Africa, and South America.

Similar to ST1, ST3 has been found in human, porcine, and avian hosts. All cases identified so far have been detected in Europe, Asia, and Africa (Table S1). Seven articles include sequence data on ST3 submitted to GenBank (the present study included), with available data pertaining to 17 samples. In the study by Sylvain et al. (20) from Cameroon, ST3 was reported in 6/80 (7.5%) *Entamoeba*-positive individuals; for two of the six cases, ST3 sequences were deposited in GenBank (AB851494 and AB851496).

ST4 is the most commonly reported subtype so far in humans ($n = 33$) (Table S1). It should be noted that most ST4 infections identified so far have been detected in samples submitted for analysis in Sweden. However, ST4 has been found mainly in patients of non-Swedish origin and with a recent history of traveling, and it is also known that ST4 might be common in humans in Vietnam (2). Until very recently, ST4 had been detected only in human hosts. However, Tuda et al. (21), found ST4 in 75 Celebes crested macaques (*Macaca nigra*) in Tangkoko, North Sulawesi, Indonesia. In that study, ST4 appeared to be a practically consistent finding in these animals, being detected in 97.4% of these monkeys. Regarding the study by Tuda et al. (21), it remains unclear whether all cases of ST4 were confirmed by sequencing or whether the subtype-specific PCR designed for the study (see above) was used diagnostically. Nevertheless, at least one sample from a crested macaque was identified molecularly as ST4 (Table S1). In that study (21), a species-specific PCR was developed for the detection of ST4. However, the forward primer Epo-IMN-F (5′-AGG ATT TGT TTA ATA CAG-3′), which was apparently based on the sequence with GenBank accession no. LC082304, has less than 100% similarity to ST4 sequences produced in other studies, and the results of PCR using those primers should therefore be interpreted with some caution.

So far, cases of mixed-subtype carriage have not been identified in humans. Meanwhile, in a rhea and in pigs, examples of mixed-subtype carriage (ST1 and ST3) have been documented in the present and other studies (2, 7).

Regarding the human *E. polecki*-positive samples so far tested in Sweden (Table 1 and Table S1), and for which microscopy data are available, it should be noted that although other parasites are often found, no species of *Entamoeba* other than *E. polecki* were detected in these samples. It should also be pointed out that samples positive by PCR for *E. histolytica* or *E. dispar* were not additionally screened for *E. polecki*, and so the extent of mixed infection/colonization remains obscure; this point was also made by Verweij et al. (12).

In Sweden, 29 cases of human colonization by *E. polecki* have been confirmed to date by molecular methods. Subtypes 1, 2, and 3 have been detected in Swedish wastewater samples, while ST4 is yet to be detected in such environmental samples in Sweden. It is therefore likely that the *E. polecki* DNA detected in Swedish wastewater reflects nonhuman *E. polecki*. "UNE6" may represent the only documented human case of *E. polecki* in a Swedish patient with no recent history of traveling (2). The patient submitting the UNE6 sample was a farmer; as the sample was positive for *E. polecki* ST3, this case might reflect a case of zoonotic transmission.

With regard to the clinical microbiology relevance and perspective of *E. polecki*, many questions remain unanswered. Although considered nonpathogenic by most, this assumption is based on restricted evidence, and the clinical and public health significance of *E. polecki* carriage deserves further scrutiny. Increased insight into the clinical impact of *E. polecki* carriage could be obtained by performing molecular epidemiological surveys of human cases, with surveys that also include clinical data and data on coinfecting/cocolonizing organisms. Moreover, it remains unknown exactly when to expect *E. polecki* in humans. Over a period of 12 years, *E. polecki* has been confirmed in 29 patients, for whom samples were sent to Public Health Agency of Sweden for *Entamoeba* species differentiation. Interestingly, 23/28 cases subtyped belonged to ST4, and at least 15 of these reflected patients with a recent history of traveling to Africa or Asia; in the remaining cases, information on travel was unavailable. However, it should be noted that five of these cases reflected a family cluster.

A picture emerges where subtypes 1 and 3 are seen in human primates, pigs, and birds, while subtypes 2 and 4 are only seen in human and nonhuman primates. While host specificity is still unclear on the basis of the relatively limited amount of data, the phylogenetic relationship between the four lineages (Fig. 1) lends support to this hypothesis, where sequences belonging to ST1 and ST3 cluster together, while sequences belonging to ST2 and ST4 cluster together.

To date, *E. polecki* is the only *Entamoeba* species found in humans producing uninucleated cysts. However, uninucleated *Entamoeba* cysts found in nonhuman primate stool may reflect ribosomal lineages other than *E. polecki* ST2 or ST4. Indeed, Stensvold et al. (2) recently reported on a separate ribosomal lineage of *Entamoeba* (namely *Entamoeba* RL3) found in langurs and lutungs in a UK zoo. As evidenced by data by Regan (17), such lutungs may also harbor *E. polecki* ST2 (Table S1), and so nonhuman primates may carry at least three different *Entamoeba* lineages producing uninucleated cysts. Similarly, uninucleated cysts from pigs may reflect both *E. polecki* ST1 and ST3 as well as *E. suis*, and pigs can be positive for both *E. polecki* ST1 and *E. suis* (22, 23).

It should also be noted that limited data are available from studies on *E. polecki* in environmental samples; nevertheless, three of the four subtypes have been identified in such samples (reference 23, the present study, and M. Calusinska, X. Goux, and M. Fossepree, unpublished) (GenBank accession no. KU658421). The subtype not identified in such samples so far is ST4. While geographical distribution and host specificity remain areas of great uncertainty due to the limited number of observations and restricted data availability, ST4 might be a subtype mostly, if not exclusively, adapted

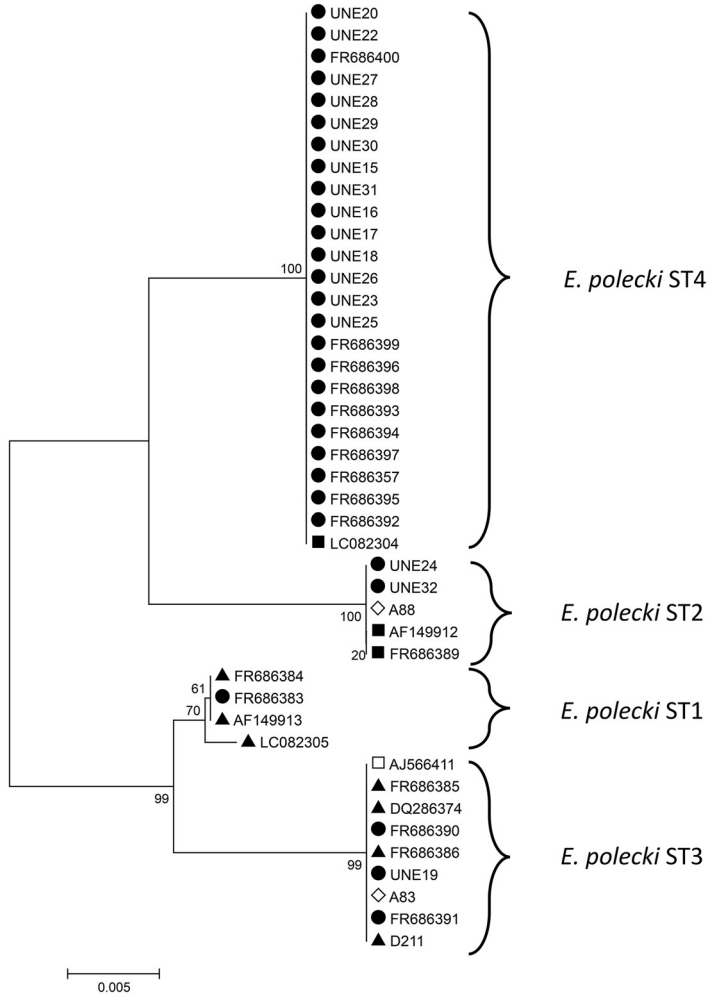

FIG 1 Phylogenetic tree showing the relationships among the samples identified in the present study (UNE samples) along with reference sequences reflecting the same region of the nuclear small subunit (SSU) rRNA gene. Most samples belonged to ST4 and were of human origin. The figure shows the trend in host specificity across the four subtypes, with ST2 and ST4 primarily found in human and nonhuman primates and with ST1 and ST3 exhibiting less strict host specificity. ●, human sample; ■, nonhuman primate sample; ▲, pig sample; □, bird sample; ◇, wastewater sample. The evolutionary history was inferred using the neighbor-joining method (24). The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. The evolutionary distances were computed using the Kimura 2-parameter method (25) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). All ambiguous positions were removed for each sequence pair. There were a total of 505 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (10).

to human and nonhuman primates. Meanwhile, other subtypes may have a more cosmopolitan distribution, probably with nonhuman hosts as the primary hosts.

On the basis of the number of complete and partial nuclear SSU ribosomal DNA sequences of *E. polecki* currently available for study, we were not able to identify any cryptic host specificity for those subtypes that appear to have relatively low host specificity. A few single nucleotide polymorphisms (SNPs) can be identified across all subtypes, but these might be due to sequencing/sequence editing errors, and none of these has been found in more than one sample. It should be taken into consideration that most sequences cover only a few hundred base pairs; therefore, any variation in other parts of the gene is missed.

In laboratories using only PCR to screen for and differentiate between *E. histolytica* and *E. dispar*, information on any other *Entamoeba* species present in a sample will not

be available. In those laboratories in which detection and differentiation of *Entamoeba* spp. still rely on microscopy, cases of *E. polecki* infection may also be overlooked, simply due to the fact that there is significant morphological overlap between immature cysts of *E. histolytica* and *E. dispar* and mature cysts of *E. polecki*. Now that a primer pair for the detection and differentiation of *E. polecki* subtypes has been evaluated and found to be highly applicable, we encourage the use of these primers in molecular surveys aiming to further clarify the epidemiology and clinical significance of these species and to solve differential diagnostic issues. Moreover, wherever possible, we encourage the sequencing of the complete nuclear SSU rRNA genes of strains within all subtypes and from various hosts with a view to identify genetic variation that might reflect cryptic host specificity.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JCM.00154-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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REFERENCES

1. Stensvold CR, Suresh GK, Tan KS, Thompson RC, Traub RJ, Viscogliosi E, Yoshikawa H, Clark CG. 2007. Terminology for *Blastocystis* subtypes–a consensus. Trends Parasitol 23:93–96. https://doi.org/10.1016/j.pt.2007.01.004.
2. Stensvold CR, Lebbad M, Victory EL, Verweij JJ, Tannich E, Alfellani M, Legarraga P, Clark CG. 2011. Increased sampling reveals novel lineages of *Entamoeba*: consequences of genetic diversity and host specificity for taxonomy and molecular detection. Protist 162:525–541. https://doi.org/10.1016/j.protis.2010.11.002.
3. Salaki JS, Shirey JL, Strickland GT. 1979. Successful treatment of symptomatic *Entamoeba polecki* infection. Am J Trop Med Hyg 28:190–193. https://doi.org/10.4269/ajtmh.1979.28.190.
4. Levin RL, Armstrong DE. 1970. Human infection with *Entamoeba polecki*. Am J Clin Pathol 54:611–614. https://doi.org/10.1093/ajcp/54.4.611.
5. Matsubayashi M, Murakoshi N, Komatsu T, Tokoro M, Haritani M, Shibahara T. 2015. Genetic identification of *Entamoeba polecki* subtype 3 from pigs in Japan and characterisation of its pathogenic role in ulcerative colitis. Infect Genet Evol 36:8–14. https://doi.org/10.1016/j.meegid.2015.08.030.
6. Clark CG, Diamond LS. 1991. Ribosomal RNA genes of 'pathogenic' and 'nonpathogenic' *Entamoeba histolytica* are distinct. Mol Biochem Parasitol 49:297–302. https://doi.org/10.1016/0166-6851(91)90073-F.
7. Jacob AS, Busby EJ, Levy AD, Komm N, Clark CG. 2016. Expanding the *Entamoeba* universe: new hosts yield novel ribosomal lineages. J Eukaryot Microbiol 63:69–78. https://doi.org/10.1111/jeu.12249.
8. Lebbad M, Ankarklev J, Tellez A, Leiva B, Andersson JO, Svärd S. 2008. Dominance of *Giardia* assemblage B in León, Nicaragua. Acta Trop 106:44–53. https://doi.org/10.1016/j.actatropica.2008.01.004.
9. Stensvold CR, Lebbad M, Clark CG. 2010. Genetic characterisation of uninucleated cyst-producing *Entamoeba* spp. from ruminants. Int J Parasitol 40:775–778. https://doi.org/10.1016/j.ijpara.2010.03.003.
10. Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. Mol Biol Evol 33:1870–1874. https://doi.org/10.1093/molbev/msw054.
11. Burrows RB, Klink GE. 1955. *Endamoeba polecki* infections in man. Am J Hyg 62:156–167.
12. Verweij JJ, Polderman AM, Clark CG. 2001. Genetic variation among human isolates of uninucleated cyst-producing *Entamoeba* species. J Clin Microbiol 39:1644–1646. https://doi.org/10.1128/JCM.39.4.1644-1646.2001.
13. Ponce Gordo F, Martínez Díaz RA, Herrera S. 2004. *Entamoeba struthionis* n.sp. (Sarcomastigophora: Endamoebidae) from ostriches (*Struthio camelus*). Vet Parasitol 119:327–335. https://doi.org/10.1016/j.vetpar.2003.10.022.
14. Verweij JJ, Laeijendecker D, Brienen EA, van Lieshout L, Polderman AM. 2003. Detection and identification of *Entamoeba* species in stool samples by a reverse line hybridization assay. J Clin Microbiol 41:5041–5045. https://doi.org/10.1128/JCM.41.11.5041-5045.2003.
15. Levecke B, Dreesen L, Dorny P, Verweij JJ, Vercammen F, Casaert S, Vercruysse J, Geldhof P. 2010. Molecular identification of *Entamoeba* spp. in captive nonhuman primates. J Clin Microbiol 48:2988–2990. https://doi.org/10.1128/JCM.00013-10.
16. Levecke B, Dreesen L, Barrionuevo-Samaniego M, Ortiz WB, Praet N, Brandt J, Dorny P. 2011. Molecular differentiation of *Entamoeba* spp. in a rural community of Loja Province, South Ecuador. Trans R Soc Trop Med Hyg 105:737–739. https://doi.org/10.1016/j.trstmh.2011.08.010.
17. Regan CS, Yon L, Hossain M, Elsheikha HM. 2014. Prevalence of *Entamoeba* species in captive primates in zoological gardens in the UK. PeerJ 2:e492. https://doi.org/10.7717/peerj.492.
18. Feng M, Cai J, Min X, Fu Y, Xu Q, Tachibana H, Cheng X. 2013. Prevalence and genetic diversity of *Entamoeba* species infecting macaques in southwest China. Parasitol Res 112:1529–1536. https://doi.org/10.1007/s00436-013-3299-1.
19. Jirků-Pomajbíková K, Čepička I, Kalousová B, Jirků M, Stewart F, Levecke B, Modrý D, Piel AK, Petrželková KJ. 2016. Molecular identification of *Entamoeba* species in savanna woodland chimpanzees (*Pan troglodytes schweinfurthii*). Parasitology 143:741–748. https://doi.org/10.1017/S0031182016000263.
20. Sylvain PN, Kaur U, Goyal K, Sehgal R, Paul MF. 2015. Molecular differentiation of *Entamoeba* spp. isolated from Cameroonian human immunodeficiency virus (HIV) infected and uninfected patient. J Parasitol Vector Biol 7:139–150.
21. Tuda J, Feng M, Imada M, Kobayashi S, Cheng X, Tachibana H. 2016. Identification of *Entamoeba polecki* with unique 18S rRNA gene sequences from Celebes crested macaques and pigs in Tangkoko Nature

Reserve, North Sulawesi, Indonesia. J Eukaryot Microbiol 63:572–577. https://doi.org/10.1111/jeu.12304.
22. Matsubayashi M, Kanamori K, Sadahiro M, Tokoro M, Abe N, Haritani M, Shibahara T. 2015. First molecular identification of *Entamoeba polecki* in a piglet in Japan and implications for aggravation of ileitis by coinfection with *Lawsonia intracellularis*. Parasitol Res 114:3069–3073. https://doi.org/10.1007/s00436-015-4509-9.
23. Hirashima Y, Manchanayake T, Yano T, Kitahara S, Koreeda T, Kamimura S, Sasai K, Matsubayashi M, Shibahara T. 2017. Development of molecular diagnostic protocols for detecting three types of *Entamoeba* from diarrheal and asymptomatic pigs and environmental moist soils. Parasitol Res 116:2001–2007. https://doi.org/10.1007/s00436-017-5483-1.
24. Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425.
25. Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16:111–120. https://doi.org/10.1007/BF01731581.